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Change in serologic specificity of rat liver cells during  
carcinogenesis with p-dimethylaminoazobenzol.

by Eberhardt Weiler.

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Translated from: Zeitschrift fuer Naturforschung, 11 b: 31-38 (1956).

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It was confirmed histo-serologically with the aid of liver-specific fluorescein-labeled antibodies that the liver-specific antigen cannot be demonstrated in primary rat liver cell carcinoma.

The liver-specific antigen is localized exclusively in the parenchymal cells of the liver.

Liver tissue exposed to the carcinogen contains less liver-specific antigen than the normal liver. This is expressed in the complement fixation test by a reduced liver-specific serologic activity of the microsomes and mitochondria, and histo-serologically in the appearance of characteristic tissue islands in which the antigenic concentration is decreased in comparison to the neighboring tissue.

The degree of antigenic loss depends upon the total dose of carcinogen administered and on the latent period.

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The microsomal and mitochondrial fractions of rat liver cells contain an organ-specific antigen (Furth, Henle 1, 2). Earlier studies had shown (Weiler 3,4) that this organ-specific antigen is no longer demonstrable in cancerous cells, i.e. in primary hepatocellular carcinoma. It was the goal of the present study to investigate the action of the liver-specific

antigen more closely during treatment of rats with p-dimethylaminoazobenzol (DAB). The total dosage of DAB, the daily dose and the latent period (= time from cessation of DAB administration to sacrifice) were varied.

Two methods were available for serologic examination:

The average quantity of antigen could be determined in the complement fixation test by the titration of microsomal and mitochondrial fractions with liver-specific antiserum. The changes noted in the antigen content of the whole tissue could then be localized in a histological section with the aid of fluorescein-labeled liver-specific antibodies, according to Coons' method (5). This method was used by Marshall (6,7), Hill and Cruickshank (8), Gitlin et al. (10) and Clayton (11) in the production of autochthonous antigens

#### Methods.

The majority of rats used belonged to strain BD I of Druckrey, some were cross-bred with this strain. The administration of DAB to these animals took place in Prof. Druckrey's laboratory (15,16).

Production of particle fractions. The rats were killed with chloroform, their liver was rapidly frozen with dry ice-acetone and stored at  $-17^{\circ}\text{C}$ . After thawing, the tissue was homogenized in a glass mortar according to Potter-Elvehjem with about 10 volume parts 0.25-m sucrose solution, buffered with m/10-phosphate, pH 7.3. Cell nuclei and tissual debris were removed by centrifugation at 1,750 g for 7 minutes (the homogenate had previously been stratified with a sucrose solution of slightly higher concentration, in order to achieve more efficient

separation). After separation of the cell nucleus fraction, the supernatant was spun at 9,000 g for 10 minutes, causing the mitochondrial fraction to sediment. The remaining supernatant yielded the microsomal sediment after centrifugation for 90 minutes at 25,000 g. The so-called "fluffy layer" which lies as a loosely packed stratum above the mitochondrial sediment, and whose nature has not been clarified (e.g.l.c. 12) to date, was combined with the mitochondrial fraction. Mitochondria and microsomes were washed once by resuspension and renewed centrifugation. The suspended particles were rapidly frozen in a CO<sub>2</sub> coolant mixture and stored at -17°C.

Production of antisera. The present tests utilized rabbit antisera against liver microsomes. Previous tests had shown (13) that the serologic activity of microsomes and mitochondria is independent of the circumstance, whether tests are conducted with liver-specific anti-microsomal or anti-mitochondrial serum. However, anti-microsomal sera yielded consistently higher titers.

The rabbits were immunized with a total of 10-20 mg antigen-nitrogen intravenously, in intervals of 2-3 days over a period of 3-4 weeks. The titer of the sera usually amounted to 1:200 in the CF test with 8  $\gamma$  N antigen.

Non-liver-specific antibodies were removed from the sera by exhaustive absorption with kidney particles (1-4). The particles were prepared from rat kidney homogenates in 0.25-M sucrose solution: After removal of the cell nucleus fraction (1,750 g, 7 min), the particle fraction was obtained by centrifugation for 40 min at 25,000 g. The sera were incubated with

the kidney particles for 1 hour at 37°C and then for one day at 3°C; the precipitate as well as non-precipitated kidney particles were separated by spinning at 25,000 g for 75 min. Usually 4-5 absorptions were required until 0.1 cc antiserum failed to react with 8  $\gamma$  N kidney antigen in the CF test.

The CF test was carried out in batches totaling 1.25 cc with 2 lytic units of complement. Antiserum, antigen and complement were incubated for 30 min at 37°C; after addition of the hemolytic system, the degree of hemolysis was read following incubation for 20 and 60 min at 37°C and standing for several hours at room temperature. The results obtained after incubation for 60 min and sedimentation of erythrocytes at room temperature were consistent and are listed in Table 2. Key: 0 = complete hemolysis (= no reaction) and 4  $\gamma$  = no hemolysis;  $\frac{1}{2}$  to 3  $\gamma$  are intermediate values.

The liver-specific serologic activity of particle fractions was established in dilution series by powers of 2. The antiserum concentration remained constant and amounted to a 8-10-fold marginal concentration, i.e. that quantity of serum which yielded at least a 3  $\gamma$  reaction in combination with 8  $\gamma$  N antigen. --- Each CF test with antigenic material from pathological livers was paralleled by a dilution series with normal liver particles as a positive control (in addition to the usual controls).

Histological demonstration of the liver-specific antigen was carried out according to the method described by Coons and coworkers (6). The fluorescein isocyanate used in labeling came from nitrofluorescein isomers designated by Coons as fraction I. The globulin fraction was used for

attachment; it was produced by precipitation and washing with 2.05-m ammonium sulfate, followed by dialysis against buffered  $\text{K}^+$  (m/100 phosphate, pH 7.5).

Removal of non-liver-specific antibodies by absorption with kidney particles took place after labeling. Control tests with labeled normal globulin showed that this renders superfluous an absorption with acetone-dried heterologous tissue powder, as prescribed by Coons.

The fluorescein-globulin was compressed to about  $\frac{1}{4}$  the original serum volume by dialysis against 20% dextrane solution (with 0.9% NaCl and m/100 phosphate). This increased the intensity of fluorescence in the section.

Cross sections were prepared through whole liver lobes. Usually the median lobe was sectioned, in most cases supplemented by cuts from the right or left lobe.

The tissue pieces were removed immediately after the animals' death, frozen in test tubes with  $\text{CO}_2$  coolant and stored in the deep freeze. They were frozen solid on the platform of a Leitz freezing microtome for the preparation of sections, without previous thawing. The microtome was installed in a box whose internal temperature was kept at  $-15$  to  $-18^\circ\text{C}$  by solid  $\text{CO}_2$ . The microtome's cutter was equipped with a plexiglass "window" on which the sections were spread out; it approximated the prototype described by Coons. The microtome was adjusted to a cutting thickness of  $5 \mu$ . The sections were transferred to slides in the freezer, the slides were then taken from the box, thawed out and dried in the cooler under a fan at  $+3^\circ\text{C}$ . They were then fixed in absolute alcohol at  $-5$  to



-10°C for a few minutes and again dried under the fan. Further treatment was carried out exactly according to Coons: Incubation for 1 hour with fluorescein-antiserum-globulin, washing in buffered NaCl for 15 minutes, and inclusion in buffered glycerol-NaCl solution.

Observation and photography was performed by means of Reichert's large micro-fluorescence apparatus, with Hg maximal pressure lamp HBO 200. For photographic purposes, the beam passage above the ocular was modified by the addition of a second UV block filter and a light yellow-green filter (in addition to the Euphos filter supplied by Reichert). Exposure time with Adox KB-17 film (Leica) was 10-12 minutes.

The following controls listed by Coons were carried out: 1. Treatment of heterologous tissue (spleen, kidney) with fluorescein-antiserum-globulin; 2. Treatment of liver sections with fluorescein-labeled normal serum-globulin; 3. Pre-treatment of liver sections with unlabeled anti-serum-globulin, followed by incubation with fluorescein-antiserum-globulin. Controls 1 and 2 showed no green fluorescence; control 3 gave traces of fluorescence. This proves that green fluorescence is based on a specific antigen-antibody reaction. Control 3 also shows that the antigen in the section is nearly quantitatively saturated by antiserum-globulin; this means that the intensity of green fluorescence in liver sections is a measure of antigen present at every point of the section.

A second section that followed each preparation treated by the method just described, served for histological comparison. It was fixed in Bouin or Carnoy immediately after thawing and stained with hematoxylin-eosin or with 1% toluidine blue solution. The specificity

of the toluidine blue stain for ribonucleic acid was tested on a number of sections with ribonuclease, n-HCl (60°) or cold perchloric acid (24 hours).

#### Results.

Complement fixation tests. Groups of 3 to 4 rats received variable total doses of DAB in daily doses of 2 or 6 mg and were killed after latent periods of different length (Table 1). The serological activities of microsomes and mitochondria from the various non-neoplastic livers with liver-specific antiserum are listed in Table 2. Particles from normal livers and particles from a hepatocellular carcinoma served as controls. The latter did not react with liver-specific antiserum, thus confirming previous results. The liver microsomes of a large number of normal rats always yielded a very uniform reaction in these tests and previous ones; the normal mitochondria, on the other hand, showed a certain amount of deviation (as in the case of 3 rats in the table). This frequently causes the difference between normal and DAB-treated rats in the case of mitochondria to be less significant than in connection with microsomes.

As is evident from the table, the serologic activity of microsomes from 200 mg DAB rats (2 mg daily, 14-day latent period) seems to be slightly reduced when compared to normal microsomes (group I). However, the other 200 mg group (6 mg daily, 110-day latent period) failed to indicate a deviation from normal (group II).

Microsomes and mitochondria from 300 mg rats (2 mg daily, 16-day latent period) have a slight, but distinctly reduced activity compared

to the normal value (group III). Both fractions of 4 rats ingesting a total dose of 350 mg (6 mg daily, 100-day latent period) have considerably less antigen (group V). Oddly enough, the activity of particles from 3 rats fed the same DAB dose, but killed after a 3-fold latent period (group VI), is exactly as great as that of normal particles. Apparently a restitution of the liver-specific antigen has taken place here during the prolonged latent period. (It should be mentioned here that previous tests (13) revealed a distinctly lowered activity of the microsomal fraction even after 450 days in isolated rats receiving a total dosage of 500 or 700 mg DAB).

The rats treated with 690-690 and 1,000 mg DAB, respectively (groups VII and VIII) yielded particle fractions with strongly reduced liver-specific antigen. Part of the animals in these groups (and one of group VI) had tumors.

Histo-serological tests. Whereas the Cf test with particle fractions can yield only an average value for the antigen content of all prepared cells, the following tests allowed the estimation of antigenic content for each individual cell relative to other cells in the section.

Examination of normal liver sections shows that the liver-specific antigen is located exclusively in the hepato-parenchymal cells (Fig. 1\*). Connective tissue and bile duct cells have a weak, whitish-blue autofluorescence (Fig. 1, lower left), which is exhibited by all tissue elements of untreated sections; its color differentiates it unequivocally from the intensely green fluorescence of the antibodies.

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\*) Fig. 1-12, see Table p. 32 b and c.

The elastica of the vessels radiates a shining white-blue. Here, too, a confusion with the fluorescence caused by the antigen-antibody reaction is impossible for the same reason.

The cell nuclei appear black in sections treated with fluorescein-antiserum. This does not, however, permit the conclusion that no liver-specific antigen is present in the cell nuclei. If the sections are fixed after treatment with fluorescein-antiserum and then stained with hematoxylin-eosin, the cells reveal, at most, only traces of basophilia. Consequently, a large part of the cellular material had been removed during the section's incubation or subsequent washing. The question, whether liver cell nuclei also contain the organ-specific antigen of the cytoplasmic particles, therefore remains unresolved.

Six hepatocellular carcinomas and one cholangioma failed to show green fluorescence with liver-specific fluorescein-antiserum-globulin (Fig. 11). The tumor sections merely reveal the usual weak white-blue auto-fluorescence in the neoplastic tissue, and isolated granular, orange-yellow fluorescent pigment deposits in the connective tissue. The earlier discovery, that hepatomas do not contain demonstrable liver-specific antigen, was thus confirmed by this method.

The gradual abatement of liver-specific antigen in precancerous livers, as established in CF tests, was expressed also histologically. While the green fluorescence of liver-specific antibodies is evenly distributed over all parenchymal cells in normal liver tissue, there are islands of tissue with reduced fluorescence in the liver of DAB-treated animals which contrast against the remaining brightly

fluorescent tissue. These islands consequently have a lower concentration of liver-specific antigen than the surrounding tissue.

The size of these islands with low antigenic content is very variable: From small cell foci (Fig. 2,4), the smallest consisting of only about 20 cells in the section, to areas which may reach across half a normal liver lobule (Fig. 5 and 6, 7 and 8).

The degree of antigen depletion in the individual islands in comparison to the surrounding tissue was variable. There were intermediary stages between barely distinguishable spots and areas that no longer contained any antigen. Only relatively small divergences from normal were demonstrable after small doses of DAB, whereas later stages of DAB treatment usually were accompanied by the presence of all gradations in one section. The degree of antigen depletion within the individual islands usually was uniform.

Instances of isolated cells with low antigenic content were not seen. Even though there were rare occasions of antigen-depleted cells completely surrounded by others rich in antigen, they nevertheless were located so close to an antigen-depleted focus of cells that their kinship with the latter cannot be doubted (Fig. 4). On the other hand, the inclusion of isolated antigen-rich cells in an antigen-depleted island was a fairly frequent occurrence.

It was frequently observed in the early stages of carcinogenesis that the cells in a certain region around the central veins were exempt from loss of antigen (Fig. 3). Large antigen-depleted spots appeared preferably around Glisson's triangles. In these spots the antigen was

consistently reduced at a relatively lower rate, and the outlines of the spots seem to be somewhat diffuse. Larger doses of DAB or longer latent periods usually produced tissue islands that were sharply delineated against the surrounding tissue and normally did not indicate topographic correlation to blood vessels (Fig. 4,5). In later stadia of DAB action, islands poor in antigen were often proximal to fibrous connective tissue proliferations or, at times, were separated from antigen-rich tissue by connective tissue lamellae (Fig. 7,8).

Rats developing a hepatoma regularly had more numerous antigen-depleted islands in the non-neoplastic liver tissue, and the antigen loss was more advanced than in animals of the same feed group that failed to grow tumors.

The histological picture of the antigen-depleted islands was not uniform. In early stages of DAB action, the islands could barely be differentiated structurally from the surrounding tissue. Pronounced histological differences appeared later: Whereas the trabecular structure in the antigen-depleted islands usually was well preserved in the early stages (Fig. 3), later stadia revealed a more irregular nature (Fig. 5 and 6, 9 and 10). Sometimes the antigen-depleted cells were smaller than normal cells (Fig. 5 and 6).

The majority of islands of antigen deprivation were more basophilic than normal tissue. This is shown in Fig. 7 and 8 with the aid of the toluidine blue stain. Control tests in which the sections were pre-treated with ribonuclease or with 1-n HCl (60°C, 15 min) prior to toluidine blue staining, indicated that the cytoplasmic basophilia was

due to the presence of ribonucleic acid.

The results of the individual groups are consolidated in Table 3. The estimated degree of antigenic reduction in the darkest islands of the various sections is calibrated 1 to 4. 1 designates a difference in fluorescence between the islands and the surrounding tissue that is just on the border of being distinctly perceptible, while livers marked 4 also contained islands with practically no antigenic content. --- With respect to the number of antigen-depleted islands, the designation "isolated" means that about 2 to 5 islands were identified in a cross section through a liver lobe, giving the cross section a potential mosaic appearance (following larger doses of DAB).

#### Discussion of results.

Serologic examination resulted in the discovery the cell-specific antigen of microsomes and mitochondria from hepato-parenchymal cells of the rat cannot be demonstrated in primary hepatomas. Liver-specific antibodies react neither with tumor particles in the CF test or in the precipitin test, nor do they produce a reaction in tumor sections (as labeled antibodies). Moreover, tumor particles injected into rabbits fail to produce liver-specific antibodies (1,13). On the other hand, antigens that do not occur in the normal liver were demonstrated in cytoplasmic particles from hematogenous tissue. These antigens are not tumor-specific, however, but are also contained in the spleen and in the kidney, partly also in the lung, heart and testes (1,13).

Particle fractions from livers subjected to the carcinogen contained less liver-specific antigen than particles from normal livers. In this

connection the liver sections revealed characteristic tissue islands in which the antigen was present in lower concentration than in the surrounding tissue. These antigen-depleted islands seem to be transitory stages in the process of complete elimination of the antigen. The decrease in antigenic activity of the particle fractions as well as the extent of histologically localizable antigen reduction become more pronounced with increasing doses of DAB.

The decisive question is whether the loss of cell-specific antigen is related causally to tumor formation. According to existing results based on DAB-induced hepatomas, the loss of antigen and the formation of tumors are closely connected temporally and topographically. No tumors were observed that still contained liver-specific antigen; non-neoplastic tissue contained small, isolated cellular islands in which no antigen whatsoever could be found (antigen reduction 4 %). In these cases a hepatoma had invariably developed at a different site in the liver, and the antigen-free islands had the appearance of microscopic carcinomas.

If antigenic loss and tumor formation are causally connected, the same laws of DAB action must be valid both for the elimination of antigen and the development of tumors. Especially the irreversibility of the process as a result of DAB's summation effect (Druckrey 14-16).

If the antigenic activity of microsomal and mitochondrial fractions is considered separately, this apparently is not the case; after 270 days of latency (350 mg DAB) there was a distinct restitution of the fraction's antigen content when compared to the effect of the same DAB dosage after a 100-day latent period. Histologically the livers still contained islands



even after the protracted latent period, in which the antigen was reduced in comparison to the surrounding tissue as strongly or more strongly than after the shorter latency. This discrepancy may be explained hypothetically by the circumstance that DAB may act in different ways on the antigenic content of the cells: For one, it reduces the antigen concentration diffusely in the whole tissue; this process (in connection with small doses of DAB) has the major role in the reduction of serologic activity in the fractions, but is impossible or difficult to recognize histo-serologically and is reversible. For another, it produces the sharply delineated antigen-depleted islands in which antigenic loss is irreversible; however, these islands represent only a relatively small share of the total tissual volume and for this reason do not have a marked effect on the activity of the particle fractions. Only the islands lead to cancer, and they are also affected by the summation effect of DAB.

A brief attempt shall be made to interpret the circumstance that the decrease in liver-specific antigen occurs in cell foci or tissue islands. This could be due to the possibility that the carcinogen is transported by the blood circulation into certain tissue areas where it acts in higher concentrations. Or the cause could be found in a primary change affecting individual cells, which only becomes evident serologically when the cells have gone through a number of divisions and have produced the foci in this manner. The fact that the first interpretation has a certain amount of validity is suggested by the finding in early stages of carcinogenesis that the antigen-depleted tissue often is located

around Glisson's triangles and that the areas around the central nerves are spared (Fig. 3). The results cannot be explained by means of this interpretation alone, however, and this for the following reasons:

1. The antigen-depleted tissue islands frequently fail to indicate a topographical relationship to the blood vessels, especially in later stages.
2. The fact that the degree of antigen reduction within the islands usually is quite uniform and that the islands are so sharply delineated against the surrounding antigen-rich tissue, cannot be explained by a concentrational gradient surrounding the blood vessel. One would expect a steady gradient of antigen depletion away from the blood vessel.

Both processes seem to be involved: The relatively diffuse spots with a low degree of antigenic loss, situated around Glisson's triangles and sparing the central veins, are a direct outgrowth of the abatement in the effective DAB concentration (Fig. 3). And the sharply delineated islands with pronounced antigenic loss are due to the fact that antigen depletion is manifested only after the primary transformation of individual cells has experienced a certain number of cell generations. Intense regenerative processes seem to take place in the liver during DAB administration, so that the formation of islands from individual cells by focal regeneration is possible. Daoust (17) found the incidence of mitosis of hepato-parenchymal cells during 150 days of DAB ingestion to be an average of 10 times greater than ordinarily encountered in the normal liver; a maximal rate of mitosis approaching a 20-fold increase was reached after 90 days of DAB (about 6 mg daily).

The relatively diffuse islands of low-grade antigen depletion which surround Glisson's triangles, are numerous in rats subjected to a total dosage of 300 or 350 mg DAB and a short latent period (groups III and IV). (Group III has the only histo-serologically demonstrable change). They are absent from group VI, with 270 days latency. This suggests that these changes are an expression of the diffuse antigenic loss which was proved reversible in the CF test.

The biological process on which the transformation of cellular antigens during carcinogenesis is based, is unknown. The involvement of a "somatic mutation" is unlikely, since histo-serologic appearances indicate a continuous process of antigen elimination, which extends over a long period of time. One would expect a less constant behavior from a mutation. It seems plausible that the process of cell antigen transformation is related to the activity that leads to cellular differentiation during embryonal development and concurrent formation of cell-specific antigens.

#### Illustrations.

Fig. 1-4, 5, 7, 9 and 11 are fluorescence photographs of liver sections treated with liver-specific fluorescein-antiserum-globulin. Magnification 160:1.

Fig. 1. Normal liver.

Fig. 2. 200 mg DAB (group II). Island with antigen reduction 1, bordering on a blood vessel.

Fig. 3. 350 mg DAB (group V). Islands with antigen reduction 1.

- Fig. 4. 350 mg DAB (group VI). Island with antigen reduction 2.
- Fig. 5. 690 mg DAB (group VII). Part of an island with antigen reduction 3.
- Fig. 6. Same field as Fig. 5, hematoxylin-eosin.
- Fig. 7. 1,000 mg DAB (group VIII). Part of an island limited by a connective tissue lamella. Antigen reduction 2.
- Fig. 8. Same field as Fig. 7, toluidine blue stain.
- Fig. 9. 1,000 mg DAB (group VIII). Island with antigen reduction 3.
- Fig. 10. Same field as Fig. 9, hematoxylin-eosin.
- Fig. 11. Edge of a primary hepatoma (from group VIII).
- Fig. 12. Same field as Fig. 11, hematoxylin-eosin.

Group	Total DAB dosage (mg)	Daily DAB dosage (mg)	Latent period (days)
I	200	2	14
II	200	6	110
III	300	2	16
IV	300 or 350	6	14
V	350	6	100
VI	350	6	270
VII	650 or 690	6	26
VIII	1000	6	10

Table 1. Data of DAB administration.

Table 2. Complement fixation tests with cytoplasmic particles from non-neoplastic, DAB-treated livers with liver-specific antiserum. Antiserum excess. Antigen dilution series for each individual rat. For DAB treatment of the various groups, see Table 1.

Feed group	Number of animals	Maximal antigen reduction	Relative dimensions and relative number of antigen-depleted islands	Remarks
I	3	-	-	No distinct divergence from normal
II	3	1 to 2	Small, isolated	Fig. 2.
III	3	1 to 2	Large, isolated	as Fig. 3, diffuse delineation
IV	5	2 to 3(*)	Small and large, numerous	Large islands diffuse: Fig. 3; small islands defined: as Fig. 4.
V	3	2 to 3	Small and large, numerous	Large islands diffuse: Fig. 3; small islands defined: as Fig. 4.
VI	1	3	Small, numerous	Hepatomatous animal. Islands defined.
	2	2 to 3	Small, isolated	Islands defined: Fig. 4.
VII	3	3 to 4	Small and large, numerous	2 hepatomatous, 1 angiomatous. Islands defined: Fig. 5 and 6.
	2	3	Small and large, numerous	Islands defined.
VIII	3	3 to 4	Small and large, numerous	3 hepatomatous animals, islands defined: Fig. 9 and 10.
	3	3 to 4	Small and large, numerous	Islands defined: Fig. 7 and 8.

(\*) 1 animal had only antigen reduction 1.

Table 3. Histo-serologic findings in non-neoplastic, DAB-treated livers. For explanation, see text.

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